IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

DECLARATION UNDER 37 CFR §1.132

Honorable Commissioner of Patents

Sir:

I, Priit Kogerman, an inventor of the above mentioned patent application declare and state as follows:

After earning my Ph.D. in tumor biology at Case Western Reserve University, OH, USA in 1997 I moved to Sweden the same year to take up a postdoctoral position in Karolinska Institutet, Deparmten of Biosciences. There I continued my work on tumor biology. In 1999 I was nominated as associate professor in Tallin Technical University. I now have over 10 years of experience, following my doctorate in tumor physiology and biology with focus on discovery of new generation anticancer drug candidates. My current position is Professor and Chair of Molecular Diagnostics at Tallinn University. I have published a number of refereed papers, and named as an inventor in a number of patent applications.

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As is evident from my credentials, I have an extensive experience in tumor biology and diagnostics upon which to base my opinion as a skilled artisan, and my extensive experience allows me to compare existing technologies to what is disclosed in the above mentioned application and to evaluate whether a method is obvious from prior art or not.

Since we submitted the patent application for "Obtaining and use of therapeutic antibodies entering into the cell" we have continued to conduct experiments on the invention and I am showing some of the results here as evidence that membrane permeable fusion proteins that will enter effectively into cells and retain their initial biological activity is not obvious. Specifically I want to show that without experimental data one cannot predict fusion proteins to enter the cells and retain their initial biological activity. This is evidenced by the experiment described below:

Recombinant anti-Gli3 scFv constructs composed of the VH and VL domains of a corresponding monoclonal antibody sequence (clone 5E1), connected via a flexible 19-residue linker, were cloned into pET40 vector using methods known in the art. In one construct, transportan was included in the fusion protein at its C-terminus. The proteins were expressed in *E. coli* in fusion with N-terminal bacterial chaperone DsbC and (His)$_6$-tag, and affinity-purified over Ni-NTA beads.

Below are the protein sequences of the obtained recombinant anti-Gli3 5E1 scFv constructs with and without Transportan. VH and VL domains appear in bold, linker residues in italics and Transportan sequence is underlined.

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DsbC-5E1-Tra
SEQ ID NO:1

```
  1    MKKGFMLFTL LAAFSGFAQA DDAAIQQTLA KMGIKSSDIQ PAPVAGMKTV      50
 51    LTNSGVLYIT DDGKHIIQGP MYDVSGTAPV NVTNKMLLKQ LNALEKEMIV     100
101    YKAPQEKHVI TVFTDITCGY CHKLHEQMAD YNALGITVRY LAFPRQGLDS     150
151    DAEKEMKAIW CAKDKNKAFD DVMAGKSVAP ASCDVDIADH YALGVQLGVS     200
201    GTPAVVLSNG TLVPGYQPPK EMKEFLDEHQ KMTSGKGSTS GSGHHHHHHS     250
251    AGLVPRGSTA IGMKETAAAK FERQHMDSPD LGTDDDDKSP GFSSTMAISD     300
301    PRVQLQQSGP ELVKPGASVK ISCKASGYSF TGYFMNWVKQ SHGKSLEWIG     350
351    RINPYNGDTF YNQKFKGKAT LTVDKSSSTA HMELLSLTSE DSAVYYCGRS     400
401    GYDLYAMDYW GQGTSEFSSG GGGSGGGGSG GSVDQIVLTQ SPAIMSASPG     450
451    EKVTMTCSAS SSVSSRYLHW YQQKSGASPK LWIYGTSNLA SGVPARFSGS     500
501    GSGTSYSLTI SSVEAEDAAT YYCQQYHSDP WTFGGGTKEF GWTLNSAGYL     550
551    LGKINLKALA ALAKKIL
```

DsbC-5E1
SEQ ID NO:2

```
  1    MKKGFMLFTL LAAFSGFAQA DDAAIQQTLA KMGIKSSDIQ PAPVAGMKTV      50
 51    LTNSGVLYIT DDGKHIIQGP MYDVSGTAPV NVTNKMLLKQ LNALEKEMIV     100
101    YKAPQEKHVI TVFTDITCGY CHKLHEQMAD YNALGITVRY LAFPRQGLDS     150
151    DAEKEMKAIW CAKDKNKAFD DVMAGKSVAP ASCDVDIADH YALGVQLGVS     200
201    GTPAVVLSNG TLVPGYQPPK EMKEFLDEHQ KMTSGKGSTS GSGHHHHHHS     250
251    AGLVPRGSTA IGMKETAAAK FERQHMDSPD LGTDDDDKSP GFSSTMAISD     300
301    PRVQLQQSGP ELVKPGASVK ISCKASGYSF TGYFMNWVKQ SHGKSLEWIG     350
351    RINPYNGDTF YNQKFKGKAT LTVDKSSSTA HMELLSLTSE DSAVYYCGRS     400
401    GYDLYAMDYW GQGTSEFSSG GGGSGGGGSG GSVDQIVLTQ SPAIMSASPG     450
451    EKVTMTCSAS SSVSSRYLHW YQQKSGASPK LWIYGTSNLA SGVPARFSGS     500
501    GSGTSYSLTI SSVEAEDAAT YYCQQYHSDP WTFGGGTKLA AALEHHHHHH     550
551    H
```

The resulting anti-Gli3 fusion proteins were labelled with AlexaFluor 488
fluorescent dye and tested for their ability to enter into cultured Cos-7 cells. The
proteins were applied for 30 minutes, the cells were washed and fixed, and
fluorescence microscopy was performed. Figure 4A depicts internalisation of
DsbC-5E1-Tra and Figure 4B depicts internalisation of DsbC-5E1. As can be
seen, both proteins show very low internalisation efficiency.

In addition, internalisation properties of the above proteins were tested by flow
cytometry. Cultured HEK-293 cells were incubated with the proteins for 2 hours,
washed and analysed for intensity of fluorescent signal from the cell interior. The
assay also included a control protein, DsbC-5E1 + TP10, comprising DsbC-5E1
that had been chemically conjugated to transportan TP10 peptide in a manner

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described in Example 3 of the patent application. The results are shown in Figure 4C (black = background fluorescence; red = DsbC-5E1-Tra; green = DsbC-5E1; blue = DsbC-5E1 + TP10).

I am providing this information in order to show that one skilled in the art would immediately realize that without experimental data one cannot predict the internalization of the molecule into the cell. This is an important issue that should be taken into account when determining whether existing prior art makes the instant invention obvious.

We have also performed experiments with VL-TP-Linker-VH, with linker peptides of various lenghts. We created recombinant fusion proteins taht contained linkers as tandem repeats (2xlinker adn 3x linker sequence). The results of these experiments demonstrated taht insertion of these longer sequences dramatically decreased the ability of fusion proteins to enter the cell. Most probably longer linkers hindered sterically transport peptide part of fusion protein. This is another demonstration that the instant inventions is not obvious.
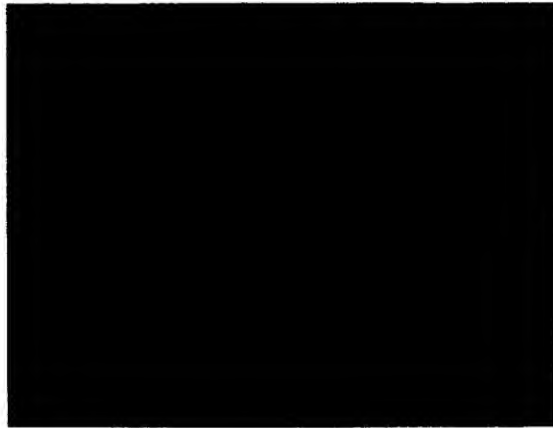
FIGURE 4A

FIGURE 4B

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I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

July 10, 2009
Date

Priit Kogerman

FIGURE 4A
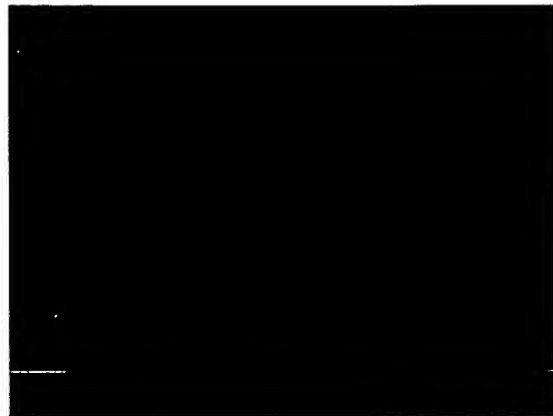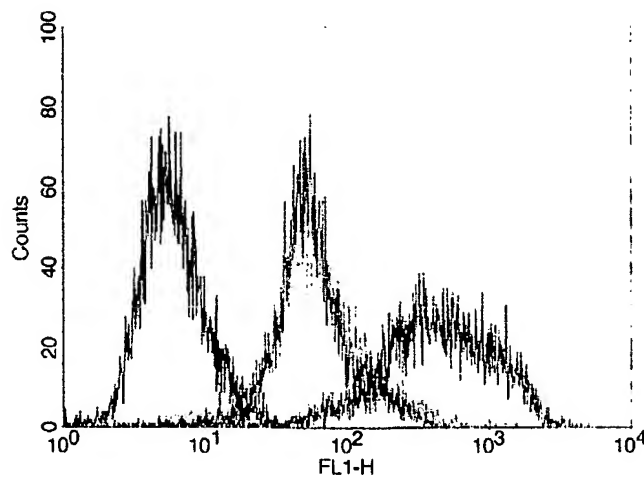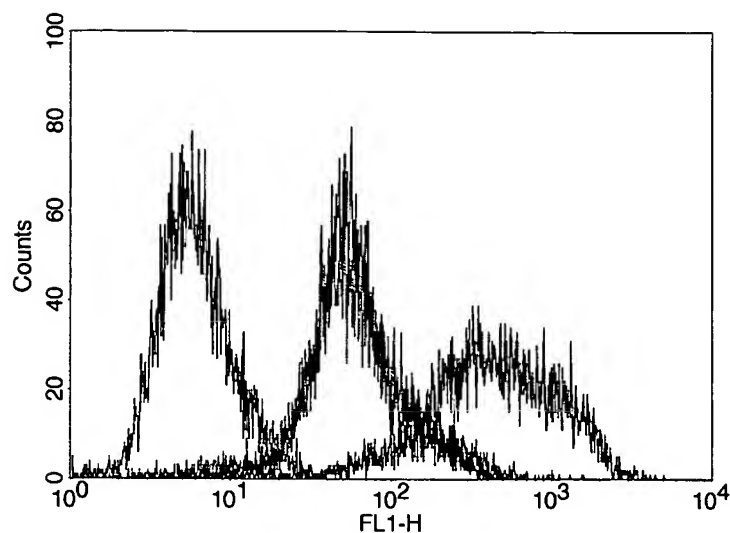

FIGURE 4B

I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

_____   _____

Date   Priit Kogerman

Priit Kogerman

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Additional information	

Career

Institution and position held	04/07/2008 - ... Tallinn University of Technology , Faculty of Science, Department of Gene Technology, Chair of Molecular Diagnostics; Lead Researcher (0.75)
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	2005 - 04/06/2008 Tallinn University of Technology , Faculty of Science, Department of Gene Technology, Chair of Molecular Diagnostics; (0.75)
	2005 - 2007 Tallinn University of Technology , Faculty of Science, Department of Gene Technology, Chair of Molecular Diagnostics; Professor (0.25)
	1999 - 2005 1999-2005 Senior Research Scientist (2001- 2005 Head of the Laboratory) Laboratory of Molecular Genetics, National Institute of Chemical Physics and Biophysics
	1998 - 2005 National Institute of Chemical Physics and Biophysics; Senior Researcher (1.00)
	1991-92 researcher, University of Helsinki, Institute of Biotechnology
	1992-96 research assistant, Case Western Reserve University, Department of Molecular Biology and Microbiology
	1997-98 postdoctoral fellow (1999-2002 Assistant Professor) Karolinska Institutet, Department of Biosciences at Novum
	1999-2001 Associate Professor (2002- Invited Professor in Tumor Biology) Tallinn Technical University Gene Technology Center (2002- Department of Gene Technology)
Education	1985-88 Moscow State University, Faculty of Biology
	1988-92 University of Tartu, cum laude Diploma in Biology and Biochemistry
	1992-96 Case Western Reserve University, Ph. D. studies
Administrative responsibilities	2007 - ... Vice President, Estonian Society of Human Genetics, Member of the Gene Technology Commission, National Representative, EC Innovative Medicine Initiative; Expert, Competition of Estonian Student Science Projects, Council Member, TUT Technomedicum, ICM
	1991 - 2006 Has been/is member in different scientific societies (incl. Finnish Chemical Society, Estonian Biochemical Society, AACR, AAAS, ACS, Estonian Society of Human Genetics, Estonian Genome Foundation) Has been member of the Scientific Council and Head of

Research activity

Degree information	**Priit Kogerman**, Doctor's Degree, 1997, (sup) Lloyd Culp, PhD (PhD), Case Western Reserve University
Honours & Awards	0, Priit Kogerman; 2001: President of Estonia, Young Scientist Award 1999: Paul Kogerman Memorial Medal 1997: Visby Scholar, Swedish Institute 1995: Jüri Lellep Memorial Award, Nikolai Küttis Memorial Award 1993: Rotalia Foundation Award 1991: Rector of the Univer
Field of research	Health, Biomedicine
	Biosciences and Environment, Biotechnology, Molecular Biology, Cell Biology, Biophysics and Economic and Technological Research relating to Bio- and

	Environmental Sciences
Current grants & projects	Molecular biology of tumor progression: molecular mechanisms and biomedical applications
	Molecular biology and immunology of tumor progression: molecular mechanisms and biomedical applications
	Development of cost-effective in ovo tumor models for imaging studies (MRI, ultrasound)
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	Kristiina Tamboom, PhD Student, (sup) **Priit Kogerman**, CD44 ja PTCH-SSH raja seos rinnavähiga, **Tallinn University of Technology , Faculty of Science, Department of Gene Technology**
	Piret Tiigimägi, PhD Student, (sup) **Priit Kogerman**, Transkriptsiooni repressorid SHH-PTCH signaalirahas, **Tallinn University of Technology , Faculty of Science, Department of Gene Technology**
	Olga Bragina (Njunkova), PhD Student, (sup) **Priit Kogerman**, Transkriptsioonifaktori GLI2 uurimine, **Tallinn University of Technology , Faculty of Science, Department of Gene Technology**
	Marit Merigan, Master's Degree, (sup) **Priit Kogerman**, AFP retseptori identifitseerimine bakteriaalse kaksikhübriidi metodi abil ja sidumismeetodil cDNH raamatukogu eukarüootsetes rakkudes ekspresseerides., **Tallinn University of Technology , Faculty of Science, Department of Gene Technology**
Dissertations supervised	**Pille Pata**, Master's Degree, 2008, (sup) **Illar Pata, Priit Kogerman**, Gli transkriptsioonifaktorid Hedgehog/Wnt interaktsioonide vahendajatena (Gli transcription factors as mediators of Hedgehog/Wnt interactions), **Tallinn University of Technology , Faculty of Science, Department of Gene Technology**
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	Wally Anderson, Master's Degree, 2007, (sup) **Priit Kogerman**, Taavi Päll, CD44 valgul põhineva angiogeneesi inhibiitori toime endoteelirakkude signaaliradadele, **Tallinn University of Technology , Faculty of Science, Department of Gene Technology**
	Anne Meikas, Master's Degree, 2002, (sup) **Priit Kogerman**, Andres Valkna, G5 PAMAM-dendrimeeri ja PEI võrdlev kasutamine plasmiidse DNA ja oligonukleotiidide rakku viimisel, **Tallinn University of Technology , Faculty of Science, Department of Gene Technology**
	Silja Moik, Master's Degree, 2008, (sup) **Priit Kogerman, Illar Pata**, Hedgehog/GLI signaliseerimine glioomi rakuliinides ja GLI2 N-terminaalse ala funktsionaalne kirjeldamine, **Tallinn University of Technology , Faculty of Science, Department of Gene Technology**
Additional information	Ph. D. (Molecular Biology) Case Western Reserve University, 1997

Publications

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Inventions

Invention: Molecules associated with the human suppressor of fused gene; Owner: Karolinska Innovations AB; Authors: Priit Kogerman, Rune Toftgaard, Peter Zaphiropoulos, Thomas Grimm; Priority number: SE19970004788; SE19980002293; Priority date: 19.12.1997

Invention: Obtaining and use of therapeutic antibodies entering into the cell; Owner: InBio OÜ; Authors: Andres Valkna, Priit Kogerman; Priority number: EE20020000531; Priority date: 17.09.2002

Invention: Cell-selective delivery system; Owner: CEPEP AB; Authors: Priit Kogerman, Mattias Hällbrink, Margus Pooga, Madis Metsis, Andres Valkna, Anne Meikas, Maria Lindgren, Astrid Gräslund, Claes Göran Östensson, Metka Budihna, Matjaz Zorko, Anna Elmquist, Ursel Soomets, Pontus Lundberg, Peter Järver, Külliki Saar, Samir El-Andaloussi, Kalle Kilk, Ülo Langel; Priority number: SE20020001863; US20020391788P; Priority date: 18.06.2002

Invention: Novel inhibitor of angiogenesis; Owner: Celecure; Authors: Taavi Päll, Wally Anderson, Lagle Kasak, Anne Pink, Priit Kogerman, Aire Allikas, Andres Valkna; Priority number: US60/949,518; Priority date: 13.07.2007

Invention: New angiogenesis inhibitors based on soluble CD44 receptor hyaluronic acid binding domain (New Drug); Owner: Angitia AB, Priit Kogerman, Staffan Strömblad, Taavi Päll; Authors: Priit Kogerman, Taavi Päll, Staffan Strömblad; Priority number: SE20010002823; US20010314971P; Priority date: 24.08.2001

Invention: Novel molecules and mechanism for therapy; Owner: Tallinn University of Technology ; Authors: Robert Tsanev, Priit Kogerman, Kalju Vanatalu, Torben Osterlund, Illar Pata; Priority number: US60/980895; Priority date: 18.10.2007

ELSEVIER

Intrabodies as drug discovery tools and therapeutics
Martin Stocks

Within the biomedical and pharmaceutical communities there is an ongoing need to find new technologies that can be used to elucidate disease mechanisms and provide novel therapeutics. Antibodies are arguably the most powerful tools in biomedical research, and antibodies specific for extracellular or cell-surface targets are currently the fastest growing class of new therapeutic molecules. However, the majority of potential therapeutic targets are intracellular, and antibodies cannot readily be leveraged against such molecules, in the context of a viable cell or organism, because of the inability of most antibodies to form stable structures in an intracellular environment. Advances in recent years, in particular the development of intracellular screening protocols and the definition of antibody structures that retain their antigen-binding function in an intracellular context, have allowed the robust isolation of a subset of antibodies that can function in an intracellular environment. These antibodies, generally referred to as intrabodies, have immense potential in the process of drug development and may ultimately become therapeutic entities in their own right.

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Introduction
The terms 'intrabody' or 'intracellular antibody', as used today in the literature, actually cover two classes of molecule, namely 'true intrabodies' and 'retained anti-bodies'. Both types of intracellular antibody are retained within the bounds of the outer membrane of a cell, but the biophysical circumstances of their expression result in their being significantly different molecules. The ratio-nale behind differentiating the two types of intracellular antibody is explained in Figure 1. For the purposes of this review, the term 'intrabody' will refer only to true (cytoplasmically expressed) intrabodies.

The use of antibodies to block the function of intracel-lular processes dates back to the late 1970s, when inves-tigators found that they could block the function of target molecules by microinjecting purified antibody into indi-vidual cells [1]. Although such experiments were highly informative, for intracellular antibodies to become a broadly applicable technology for probing biological func-tion *in vitro* and *in vivo* required a means of introducing or expressing antibodies intracellularly in large cell popula-tions or in animals. Early attempts to express antibodies met with some success [2–4], but in general it was found that antibodies or antibody fragments either failed to express or did not bind their target antigen in an intra-cellular context. The explanation for this was to be found in the general structure, and biosynthetic pathway, of the antibody fragments. Antibodies are naturally extruded through the membranes of the endoplasmic reticulum, and secreted into the extracellular space, within the B-cells that produce them. Attempts to express antibody fragments in an intracellular compartment have mostly been unsuccessful, as the reducing environment, which prevents the formation of disulfide bridges, and the absence of accessory factors to help the antibodies fold, serve to prevent the antibody structure from achieving the requisite conformation [5]. To overcome these issues, scientists have had to develop both new ways of building antibody libraries and new methods of selecting from those libraries. This review is intended to outline recent advances in the field of intrabody development, from methods of intrabody generation to end use, as both research tools and potential therapeutics. I also intend to provide some educated speculation as to the potential value of intrabodies in the area of drug discovery and development.

The generation of functional intrabodies
Advances in the structural formulation of recombinant antibodies, rapid screening protocols and the creation of large unbiased libraries have made critical contributions to the development of intrabodies. In particular, the development of single-polypeptide forms of antibody sub-fragments (single-chain Fv and single domain) have been invaluable for facilitating the engineering of anti-body structures and generating synthetic libraries. How-ever, these technological advances in themselves were not sufficient for the consistent and reliable generation of individual intrabodies, and early attempts to create intra-body technology platforms stalled as the overarching issue of structural stability became apparent. In practice, the great majority of antibodies, isolated by classical methods such as hybridoma or phage-display, are unable to function when expressed in the cell cytoplasm. This is
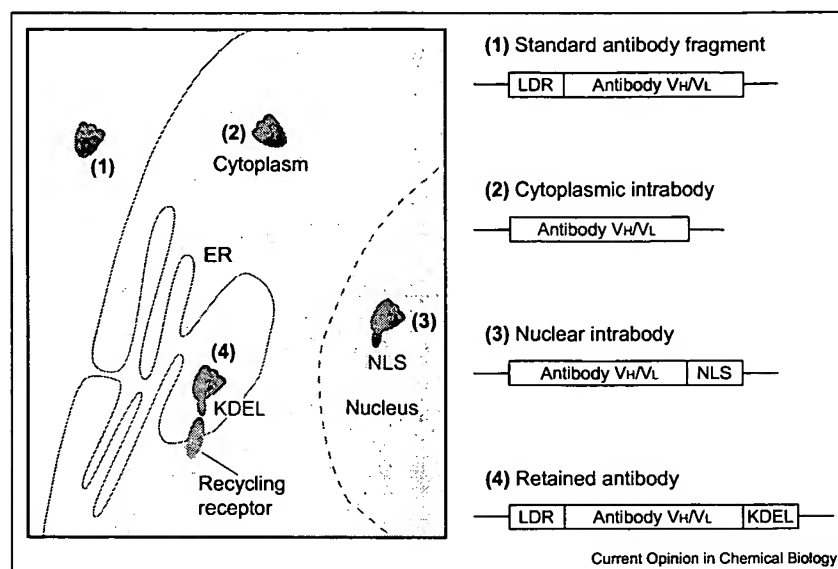
Antibodies, retained antibodies and intrabodies. In the normal course of events (1), an antibody gene begins with a leader sequence (LDR), which directs synthesis of the antibody into the lumen of the endoplasmic reticulum (ER), where the leader is cleaved away and various accessory factors assist the correct folding of the antibody and catalyse the formation of intrachain and interchain disulfide bridges that stabilize the structure. The mature antibody then exits the ER network and is free in the extracellular fluids, or exposed on the cell surface. For most applications of recombinant antibodies, the leader sequence is retained or substituted for an equivalent structure that directs antibody synthesis to the outside of the cell. For an antibody to be retained within the body of the cell, it must either be expressed into the cytoplasm or prevented from leaving the ER. Removing the leader sequence, or substituting it with a more hydrophilic amino acid group, will result in the antibody being synthesized in the cell cytoplasm (2), and the addition of subcellular localization motifs such as the nuclear localization signal (NLS) can direct the antibody to particular regions or organelles (3). These entities are 'true' intrabodies, in the sense of being topologically inside of the cell membrane. Very few antibody sequences are capable of productively folding into a functional intrabody in this way, due to the reducing environment of the cell cytoplasm. The alternative form of intracellular antibody, also referred to as intrabodies in the literature, utilizes ER/Golgi retention signals to prevent an antibody from exiting the ER after synthesis (4). The antibody fragments have a short peptide appended to their C-termini that contains the sequence KDEL, which is recognized by recycling receptor proteins within the ER/golgi membranes. Although these antibodies are never extruded or secreted from the cell, they are still topologically on the outside of the cell membrane (extracytoplamic), and benefit from the normal biosynthetic machinery within the ER to fold productively. These entities are referred to as 'retained antibodies' throughout this review.

due largely to the reducing environment within the cell, which prevents the formation of disulfide bonds that are crucial to the structural integrity of most antibody frameworks. There was therefore a need to either isolate [6**,7**,8] or develop [5,9,10,11**,12] highly stable antibody frameworks that could fold independently. The use of intracellular screening protocols [13,14**], in particular modifications of the yeast 2-hybrid system [15], to empirically isolate functional intrabodies from libraries has been instrumental in achieving this end. The creation of an empirical system for intrabody isolation that utilized successive rounds of phage-display, yeast 2-hybrid and mammalian 2-hybrid [13] ultimately led to the definition of an intrabody consensus sequence [6**,7**], which defines a subset of naturally occurring frameworks that are capable of supporting a variety of antigen-binding structures (complementarity determining regions or CDRs). A diagrammatic distribution of this intrabody consensus sequence is illustrated in Figure 2. Previously

reported intrabodies showed no structural similarities, but were serendipitous combinations of framework and CDR that happened to be capable of intracellular folding. Some effort has been made to develop intrabody libraries by taking such one-off antibodies, or stabilized derivatives, and mutating the CDRs to generate a repertoire [8,14**]. This approach carries the risk that even minor changes in the CDRs (or framework) may significantly reduce the intracellular folding capacity of the antibody, making the production of diverse CDR repertoires on such frameworks problematic. However, the isolation of several diverse intrabody specificities from such libraries have been reported [14**]. The consensus sequence, by contrast, appears to define a framework that supports a range of CDRs, while maintaining the capacity to fold in the cytoplasmic compartment. This enables synthetic repertoires to be created and screened with much greater confidence that individual clones will be functional. When combined with the intracellular screening proto-

Figure 2
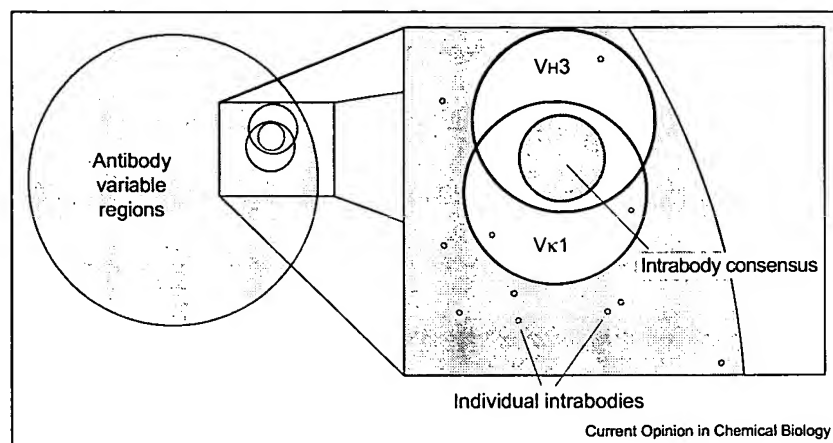
The distribution of intrabodies within the antibody variable region gene pool. The great majority of antibody variable regions are incapable of folding productively within the cytoplasm of a cell. Those that have been reported to date fall into two categories. Individual antibodies have been discovered that, through a combination of the properties of all their structural elements (both framework and antigen-binding regions), are capable of folding and retaining activity on intracellular expression. These antibodies show few or no common structural features and appear to be serendipitous intrabodies. The intrabody consensus is defined by a group of intrabodies that share common framework elements, but have widely different antigen-binding elements (CDRs), and as such appear to represent a sub-population of intrabodies within the universe of antibody variable genes. To date only the $V_H3:V\kappa1$ consensus has been reported (see text for references), but other alternative consensus intrabody sequences may exist.

cols such as the yeast or mammalian 2-hybrid systems, a highly reliable engine for intrabody generation results [16*,17].

Other methods of intrabody isolation have been reported, although their general applicability remains to be seen. Yeast surface display has been used to select a V_L single domain intrabody to the huntingtin protein [18], while others have reported that 'camelization' (mutation of certain sequence elements to make them similar to camel antibodies, which are naturally single domain in nature) of a rabbit V_H fragment resulted in an antibody having the properties of an intrabody [19]. It has also been demonstrated that by pooling large numbers of clones isolated from a very high-complexity ($>10^{10}$) phage library, and using the pools in cell-based assays, one can detect intrabody activity that can then be subsequently isolated by sequential subdivision of the pools [20]. Other approaches involve the modification of existing antibodies to enhance their intracellular stability, for instance by appending other structural elements, such as an F_C fragment [21] or the maltose-binding protein. It remains to be seen whether these techniques will provide a robust platform for intrabody generation, but it is encouraging that so much effort and invention is being applied to defining successful intrabody platform technologies, perhaps indicative of the perceived value of successful intrabody reagents.

Applications of intrabodies

The potential utility of intrabodies covers a very broad range of applications, including direct therapeutic inter-

vention [16*], target discovery and validation [17,22*], and agricultural biotechnology [23]. What immediately becomes obvious from a review of recent literature is the potential for intrabodies to find application in a diverse array of fields, particularly in almost all areas of therapeutic development. In oncology, intrabodies specific for H-RAS have been shown to block transformation in cell culture [11**,24], as have intrabodies to cyclin E [25] or the NS3 protein of hepatitis C virus [26,27]. Retained antibodies that inhibit the surface expression of the folate receptor have been demonstrated to inhibit the growth of ovarian cancer cells [28], while adenoviral delivery of an anti-tie-2 retained antibody appears to strongly inhibit tumour growth in a mouse xenograft model by inhibiting the process of angiogenesis in the tumour mass [29]. In the field of neurobiology, intracellular antibodies that can inhibit or prevent the polymerization or aggregation of candidate pathological proteins in degenerative diseases are showing some promise. Intrabodies to huntingtin have been shown to inhibit aggregation of the target protein in cell-based models of Huntington's disease [30–32], as have an anti-α-synuclein in Parkinson's disease models [33]. Retained antibodies raised against the β-amyloid precursor protein, which is involved in the pathogenesis of Alzheimer's disease, have been shown to inhibit the production of β-amyloid, either by blocking the main cleavage site or by preventing the protein from leaving the ER [34].

In transplantation, retained antibodies that prevent the export of MHC class I molecules to the cell surface are

being evaluated as a means of generating universal donor tissues [35••], while an intrabody to the retroviral p15 protein has been shown to block the production of porcine endogenous retrovirus (PERV), a major potential risk factor in the application of xenotransplantation to human therapy. Similarly, an intrabody to HIV1 Vif has been shown to significantly reduce viral replication and infectivity [19] in cells in which permissiveness is Vif dependent.

Although much of the current research focus in the intrabody field is in the area of direct therapeutic application, intrabodies also have the potential to be an extremely powerful research tool for target discovery and validation. Figure 3 shows how intrabodies might be used throughout the drug discovery process, from discovery and validation of the target molecule, to enhancing the

drug discovery process, as well as producing potential therapeutic entities in themselves. Currently, much of the target discovery and validation arena is preoccupied with RNAi [36,37••,38]. This is undoubtedly a powerful technology, and has the added advantage of being cheap and relatively easy to apply. However, RNAi is no panacea [39•,40], and intrabodies are capable of adding significant value to R&D programmes over and above what RNAi can deliver. Certain target-related factors, such as fast mRNA turnover, blocked sequence regions and slow protein turnover, can result in little or no effect of RNAi on the expression levels of the target gene-product. Gene-based knockdown is also somewhat crude in that it provides only a comparison of gene-on versus gene-off phenotypes (or more accurately, gene-high versus gene-low), whereas intrabodies offer the intriguing possibility of selectively blocking the functions of a target molecule

Figure 3
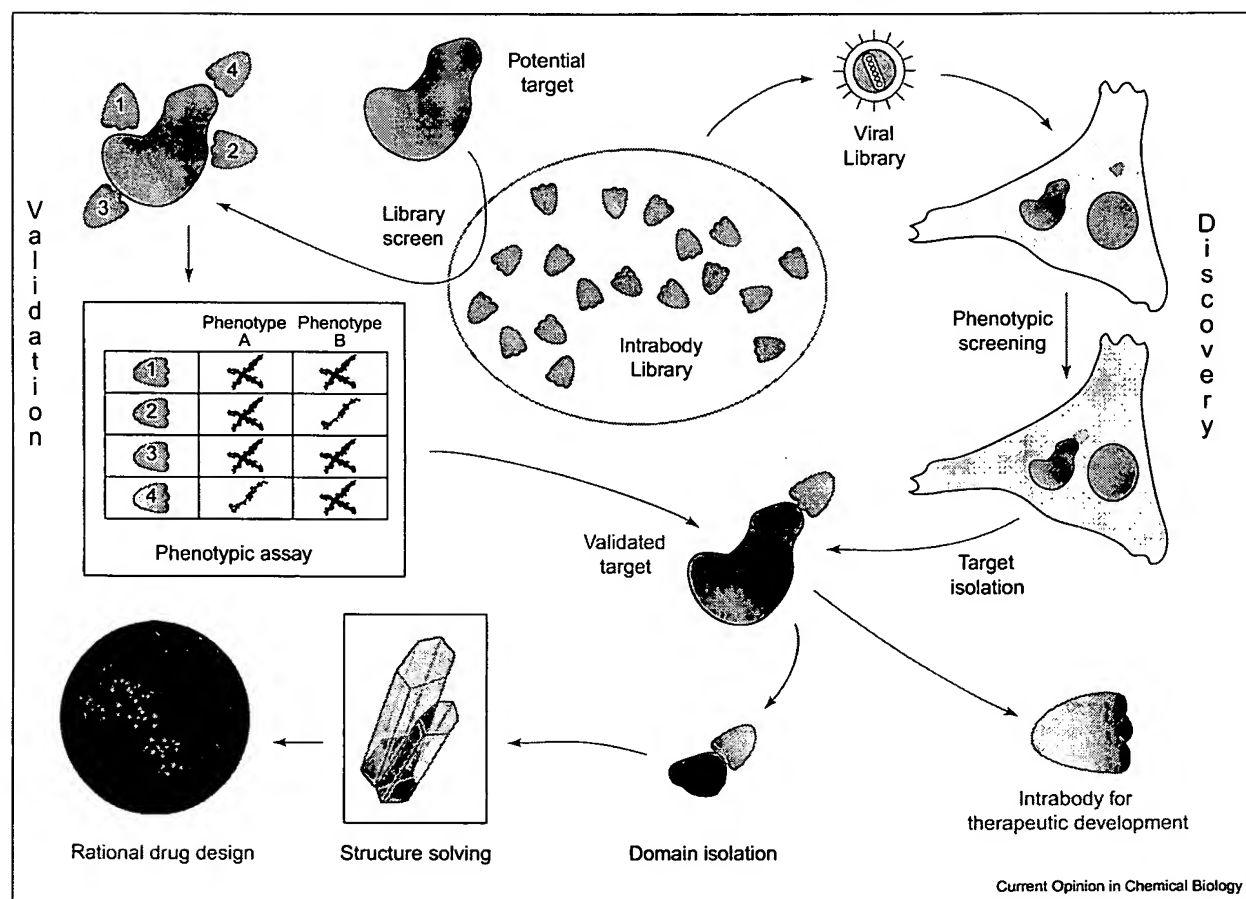
Intrabodies as an integrated platform for drug discovery and development. Intrabodies can be used at all stages of the target discovery and validation process, and can provide valuable tools for downstream drug development, or be developed as therapeutics in their own right. Because intrabodies can contact a target protein at many points, multiple phenotypic outcomes are possible with a panel of intrabodies. This cannot only produce candidate therapeutic intrabodies, but can also be of value in directing the process of small-molecule drug discovery to particular regions of the target structure. This flexibility contrasts favourably with gene-based knockdown systems for target validation that provide no more than a crude on/off readout.

on an epitope by epitope basis. Intrabodies have the added benefit of being useful biochemical tools for further analysis of a target molecule. For instance, an intrabody that produces a desired phenotype when bound to the target intracellularly can then be used *in vitro* to define critical structures and surface elements of the target by fragment selection and/or co-crystallization, thereby providing data to guide the rational design of small-molecule drugs. Such an intrabody could also be used in drug screens by searching for entities that are capable of displacing the intrabody from its target epitope, and of course the intrabody itself would be a therapeutic lead molecule.

Issues facing the use of intrabodies

Although the application of intrabodies to drug development is potentially of huge value, there remain many issues that need to be resolved before the technology becomes fully integrated into the mainstream of drug discovery and development. The critical element in making intrabodies a technology of choice for target discovery and validation is the capacity to rapidly and reliably generate panels of intrabodies to any given target. Until recently an unachievable goal, the arrival of new screening protocols and new libraries are now making such a technology platform a reality [12,17,22•]. The facility to generate intrabodies of choice also impacts upon the therapeutic application of the technology. To date, the majority of such research has been reagent-led, with the work following on from the serendipitous discovery of antibodies with intracellular capabilities. The new libraries and screens are ushering in an arena where clinically oriented work can start from a target-led approach. However, the one overarching issue for the clinical application of intrabodies is that of delivery. Fundamentally, intrabodies are a gene-based system with expression being the optimum method of getting the intrabodies into the cells and to their site of action. Although great progress is being made in the development of both viral [41] and non-viral [42] transduction systems for therapeutic application, there remains great suspicion about the whole premise of gene-based therapy, from its capacity to deliver a payload to the required sites to its general safety. Recent drug safety scares have led to the apparent abandonment of any rational risk-benefit analysis and have severely harmed the capacity of commercial entities in particular to develop gene-based technologies such as intrabodies. However, the development of technologies that might allow the direct delivery of protein therapeutics could potentially redress the balance somewhat. Advances in liposome and other encapsulation technologies are offering the potential to deliver both gene and protein to cells [43,44], and the development of protein transduction domains (PTDs) offer the possibility of intrabodies becoming an injectable drug [45••,46]. PTDs are short peptide sequences that have the ability to pass across the cell membrane, and in the process carry

with them a payload that can be anything from a short peptide to a very large protein. The technology is still in its infancy, and it is likely that individual PTDs will show considerable variability in functionality depending on both the targeted cell type and the biochemical nature of the payload. Nonetheless, PTDs represent an exciting possibility for the future delivery of many protein or macromolecular drugs.

Concluding remarks

Intracellular antibodies, either intrabodies or retained antibodies, have great potential to enhance the process of drug discovery and development. Technologies are now becoming established that should allow intrabodies to be generated with confidence, and used at will in a huge array of applications. More investment in their development is urgently required, but those companies with the vision to embrace such a powerful technology in its infancy may just find their future fortunes transformed.

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